Received: 2 June 2020 | Revised: 25 October 2020 | Accepted: 27 October 2020

DOI: 10.1111/tbed.13906

ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

First report and molecular identification of *Trypanosoma (Duttonella) vivax* outbreak in cattle population from Ecuador

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Funding information
Academy of Research and Higher Education, Grant/Award Number: Establishment of a support platform for training a; Universidad de las Fuerzas Armadas – ESPE

Abstract

The bovine trypanosomosis is responsible for economic losses from tropical and subtropical areas of Africa and Latin America. This disease is characterized by fever, anaemia, loss of production and even death. Few studies have been carried out in Ecuador regarding *Trypanosoma* spp. presence but the species has not been determined in cattle and those have only determined the presence of genus, but not the species. The aim of this study was to identify and characterize the trypanosome species involved in the suspected bovine trypanosomosis outbreak reported in Convento Village in Manabí Province located in the coastal region of Ecuador. Twenty cattle from three farms were sampled. Three samples were positive for *T. vivax*, using an end-point polymerase chain reaction (PCR) to amplify a fragment of the cathepsin L-like cysteine protease (CatL-like) gene. A phylogenetic tree analysis of these three Ecuadorian isolates showed a close relationship with isolates from South America (Colombia, Brazil and Venezuela) and West Africa (Nigeria). This is the first report of *T. vivax* in Ecuadorian cattle.

KEYWORDS
bovine trypanosomosis, cattle, Ecuador, *Trypanosoma vivax*

1 | INTRODUCTION

Protozoa of the genus *Trypanosoma* are haemotropic agents widely reported in cattle from Latin America. In this region, *Trypanosoma evansi*, *Trypanosoma theileri* and *T. vivax* are the identified species (Desquesnes, 2004). Of these, *T. vivax* is the most pathogenic, causing anaemia, fever, weight loss, reproductive problems in males and females, and low production (Gonzatti

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et al., 2014; Pereira et al., 2018; Ramírez-Iglesias et al., 2017). The mortality varies from 3% to 50%, depending on the species involved, the breed, and the immune status of the infected cattle (Desquesnes, 2004).

In South America, *T. vivax* has been reported in several countries such as French Guyana, Venezuela, Colombia, Peru, Brazil, Bolivia, Argentina and Paraguay (Gonzatti et al., 2014; Paoletta et al., 2018). Once this hemoflagellate was established in the Americas in the absence of the Tse-tse fly, *T. vivax* developed a new adaptive pathway, with modifications in the kinetoplast genome such as large deletions of the *ND7* and *COIII* genes, and frameshifts of the *ND1*, *ND2* and *ND4* genes (Greif et al., 2015).

High prevalence of *T. vivax* has been reported in Colombia (23%), determined by molecular testing (García et al., 2014); as well in two areas of the Bolivian Pantanal (27.79% and 19.03%; Gonzales et al., 2007). In Colombia, economic losses due to bovine trypanosomosis were reported to reach USD 5.2 million per year (Gonzatti et al., 2014).

In Ecuador, livestock is one of the most representative activities in the agricultural sector with approximately 4.1 million cattle distributed in the four natural regions: 48.4% in the highlands (milking cattle), 42.4% in the coastal region (dual purpose, beef/milk) and 9.13% in the jungle and Galapagos Islands (dual purpose, beef/milk). Manabí Province is located in the coastal region and contains the highest percentage of cattle (22.7%) in the country (INEC, 2019).

Because of its geographical location, Ecuador offers favourable ecological conditions for vector development, that is *Tabanus*, *Stomoxys calcitrans* and *Haematobia irritans* (Cárdenas et al., 2009). Furthermore, reports of *T. vivax* in countries in the region, including neighbours such as Colombia and Peru, suggest the probability of the presence of this hemoflagellate in Ecuador.

In July 2017, an outbreak of bovine trypanosomosis was reported in Chone Canton in Manabí Province, according to AGROCALIDAD (Regulation and Control fito-zoosanitaria Agency from Ecuador).

Bovine trypanosomosis in Ecuador is a neglected disease, like other haemotropic diseases such as anaplasmosis and babesiosis, which are evidenced in the few studies that have been carried out. However, bovine trypanosomosis seroprevalence has been reported in two studies. In a first study carried out in 1977, a seroprevalence of 22.5% was found using immunofluorescence (Wells et al., 1977). In a second study, performed 40 years later, the authors reported a prevalence of 31% using an iELISA (Medina-Naranjo et al., 2017).

According to FAO (2018), the presence of haemotropic diseases is a factor that limits efficient livestock production, reducing profitability for farmers. The aim of this study was to identify and characterize the trypanosome species involved in the bovine trypanosomosis outbreak reported in Manabí Province, using molecular methods in order to later establish specific control and treatment programs to minimize the impact of this hemoflagellate.

2 | MATERIALS AND METHODS

2.1 | Identification of trypanosomosis outbreaks

In order to identify trypanosomosis outbreaks, epidemiological reports from AGROCALIDAD and information from the Livestock Association of Convento Village were evaluated. Through this method, an outbreak of bovine trypanosomosis in July 2017 was identified in Manabí Province.

2.2 | Samples

In January 2018, the Association of Convento Village was visited, after AGROCALIDAD reported an outbreak of trypanosomosis. During this visit, only three farmers reported some cattle with clinical signs compatible with trypanosomsosis and allowed admission for veterinary evaluation. The criteria for sampling were the suspicion of the farmer about the presence of the disease in some animals and veterinary observation of clinical signs such as pale mucosa, weakness, decrease of milk production and fever.

In total, 20 blood samples were collected in tubes with EDTA from the coccygeal vein of suspected cattle: in farm UVB ($n = 6$), farm LP ($n = 10$) and farm FM ($n = 4$). Samples were temporarily stored at 4°C, until their processing and final storage at −80°C at the Laboratory of Animal Biotechnology of the Universidad de las Fuerzas Armadas ESPE, Quito, Ecuador.

2.3 | DNA extraction

DNA extraction from blood samples was performed by using the Wizard® Genomic DNA Purification Kit (Promega). DNA integrity was verified on a 0.8% agarose gel and concentration of DNA was quantified by UV spectrophotometry, using the NanoDrop 2000 kit (Thermo Fisher Scientific).

2.4 | TviCatL-PCR for diagnostic of Trypanosoma vivax

The end-point polymerase chain reaction (PCR), which amplifies a fragment of the cathepsin L-like cysteine protease (CatL-like) gene, was used to identify *T. vivax*, and the primers used were TviCatL: 5-GCC ATC GCC AAG TAC CTC GCC GA-3; and DTO155: 5-TTA AAG CTT CCA CGA GTT CTT GAT GAT CCA GTA-3, described by Cortez et al., (2009). The total reaction added up to a volume of 25 μl, composed of 1X Buffer + Cl_2Mg, 0.5 μM of each primer, 0.8 mM dNTPs, 2.5 U of Dream Taq Polymerase (Thermo Scientific) and 100 ng of DNA.

PCR was carried out in a C1000 touch thermal cycler Bio-Rad®. Cycling conditions comprised an initial activation step of 94°C for

5 min, followed by 35 cycles of 94°C for 30 s, hybridization at 65°C for 30 s and extension at 72°C for 30 s, with a final extension step of 72°C for 10 min. To verify the reliability of the results, a positive control donated by the Universidad Simón Rodríguez of Venezuela was used in each PCR round. Finally, a horizontal electrophoresis was performed using a 2% agarose gel with the PCR products stained using SYBR Safe (Life Technologies Pty Ltd), which were visualized and recorded in the ChemiDoc equipment (BIO-RAD).

2.5 | Sequencing

The amplicons of positive samples were purified using a QIAquick® Gel Extraction kit (Qiagen). Each sample was divided into three tubes and sequenced using the Sanger technique at Magrogen company in Seoul, South Korea. The nucleotide sequences obtained were aligned and edited by BioEdit v.7.2.5 (Hall, 1999) to obtain the consensus sequences, and later used to interrogate the Basic Local Alignment Search Tool (BLAST) with the database of the National Center for Biotechnology Information (NCBI). The consensus sequences evaluated were uploaded to the GenBank (Accession numbers MT547173, MT547174 and MT547175).

2.6 | Phylogenetic analysis

Phylogenetic relationships were established by analysing the catalytic domain sequences of cathepsin L-like (CatL-like) obtained in this study, with others retrieved from GenBank database and previously described (Cortez et al., 2009; Jacson et al., 2012; Jaimes-Dueñez et al., 2018) and the database (http://bioinformatica.fcien.edu.uy/Tvivax/) obtained from the analysis of the transcriptome *T. vivax* isolated LIEM- 176 from Venezuela (Greif et al., 2013). Therefore, a multiple alignment was performed using MUSCLE tool from MEGA X program (Kumar et al., 2018). Finally, the Maximum Likelihood method and Tamura-Nei model tree were performed (Kumar et al., 2018).

3 | RESULTS

The three selected farms had an extensive husbandry system, characterized by free grazing in large areas. Cattle from these farms were crossbreed *Bos taurus* and *Bos indicus*, used for dairy production. Unfortunately, the sanitary management in farms related to the use of drugs was difficult to record, because they had only an incipient record of production and reproduction.

TABLE 1 Characteristics and *Trypanosoma vivax* TviCatL-PCR results for the 20 cattle sampled in Convento Village, Manabí province, Ecuador

Farm code	Cattle identification	Animal characteristics			Clinical signs			PCR result
		Sex	Age[a]	Breed	Mucosa	T°	Haematuria	
FM	Clavel	M	36	CB	Normal	38.9	No	−
	Art 4	M	7	CB	Normal	39.5	No	−
	Paleta	F	ND	CB	Normal	39.7	No	−
	Leo	**F**	**60**	**CB**	**Pale**	**39.6**	**No**	+
LP	Abuelita	F	48	CB	Normal	ND	No	−
	Colorada	F	48	CB	Normal	ND	No	−
	Mico	M	36	CB	Normal	ND	No	−
	Beto	M	72	CB	Normal	40.9	No	−
	Chana	F	36	CB	Pale	38.8	No	−
	Mona	**F**	**84**	**CB**	**Pale**	**40.3**	**No**	+
	Estefy	F	48	CB	Pale	38.0	No	−
	Dayana	F	48	CB	Pale	39.3	No	−
	Michu	**F**	**48**	**CB**	**Normal**	**39.4**	**No**	+
	Negro pintado	M	18	CB	Normal	39.2	No	−
UVB	Panchana	M	15	CB	Normal	39.0	No	−
	Art 5134	F	42	CB	Pale	38.3	No	
	Vaca 1	F	40	CB	Pale	38.6	No	
	Café	M	15	CB	Pale	40.1	No	−
	Venado	M	12	CB	Pale	39.7	No	−
	Maduro	M	16	CB	Normal	39.4	No	−

Abbreviations: −, negative; +, positive; CB, crossbreed; F, female; M, male; ND, not determinate; T°, temperature in degree Celsius.
The lines highlighted in bold are the TviCatL-PCR positive results.
[a]Age in months.

In the veterinary evaluation, out of the 20 sampled cattle, 7 had temperatures over 39.5°C, and 9 had pale mucosa. The breed, age, sex, mucosa and temperature are recorded in Table 1.

DNA concentrations measured after extraction were in the range of 118–991 ng/μl, and the absorbance ratios A260/280 and A260/230 varied in the ranges of 1.78–1.94 and 1.80–2.4, respectively. These parameters are considered optimal for subsequent PCR analysis.

Of the 20 samples, three were positive for trypanosomes as analysed with TviCatL-PCR by amplifying a DNA 189–190 pb fragment compatible with *T. vivax* as previously described by Cortez et al., (2009). The three positive animals were found in two of the three sampled herds (Table 1).

Regarding the homology of amplicons evaluated by BLAST, we obtained 100% identity in the catalytic domain of CatL-like gene of *T. vivax* between the sequences from the used database and those obtained in this study. These sequences were recorded in GenBank with the accession numbers MT547173, MT547174 and MT547175 corresponding to samples M5, M14 and M17, respectively.

Phylogenetic analysis of the Ecuadorian isolates of *T. vivax* showed a close relation with isolates reported in South America and West Africa (Figure 1).

4 | DISCUSSION

This study demonstrated the presence of *T. vivax* in an outbreak that occurred in Convento Village in the coastal area of Ecuador, being the first scientific report of this parasite in the country. It was identified by TviCatL-PCR specific for *T. vivax* and characterized via DNA sequencing of PCR amplified product.

TviCatL-PCR of 20 samples identified *T. vivax* in 3 of them. This is evidence for the presence of *T. vivax* in the area, which suggests this parasite is the causative agent of the outbreak, together with the presence of some clinical signs compatible with bovine trypanosomosis.

The TviCatL-PCR test has turned out to be a highly sensitive and specific test in studies of genetic diversity of *T. vivax* from Africa and America (Cortez et al., 2009). This method has also been used to identify *T. vivax* in other animal species, such as wandering donkeys

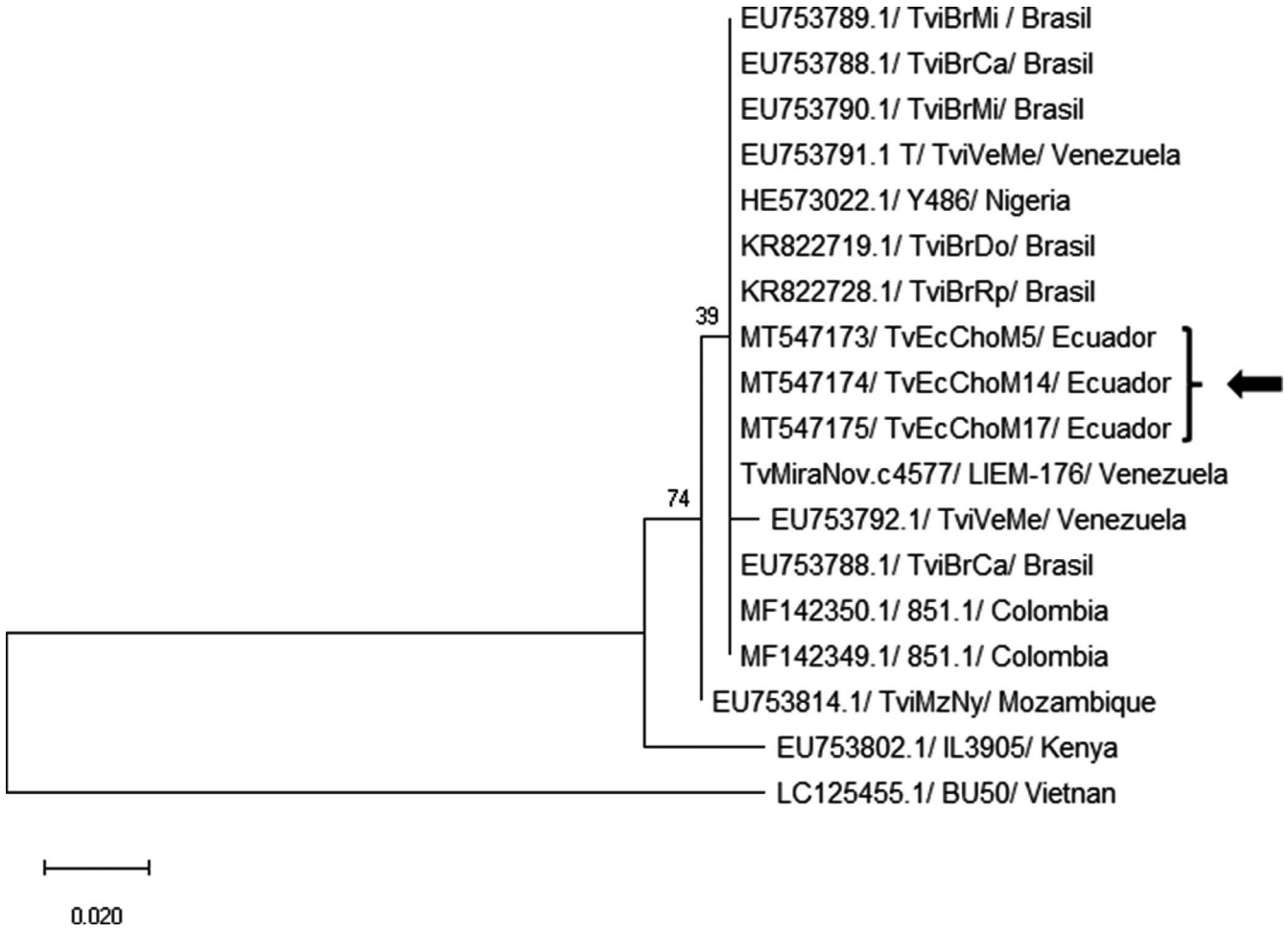

FIGURE 1 Maximum likelihood method and Tamura-Nei model tree using catL-like catalytic domain sequences from geographical isolates of *T. vivax* from Africa (Nigeria, Mozambique and Kenya) and South America (Brazil, Colombia, Venezuela and Ecuador). *T. theileri* catL was used as the out group. Taxa names consist in GeneBank code/isolates of *T. vivax*/geographical origin. Arrows indicate the Ecuadorian sequences of isolates reported in this article. The scale represents the genetic distance between analysed samples

in Brazil, where a similar genotype in cattle and sheep was found (Rodrigues et al., 2015). Likewise, other studies using PCR have been widely used, showing the capacity of the technique in epidemiological studies such as in Ethiopia (Birhanu et al., 2015).

Phylogenetic analysis of Maximum Likelihood in Ecuadorian isolates of *T. vivax* revealed a similarity with those from Western Africa and South America. The phylogenic tree showed a 100% identity for the three Ecuadorian isolates, probably because all of them are from the same outbreak and closely related to those reported in Brazil, Venezuela, Colombia, Nigeria and Mozambique, but they are separate from isolates from Kenya, which are in another clade (Figure 1). This finding has also been reported for other isolates from Latin America (Cortez et al., 2009; Jaimes-Dueñez et al., 2018).

The introduction of trypanosomosis to South America could have been produced by infected cattle from Senegal, which were brought to farms in French Guyana and Antilles (Gonzatti et al., 2014), and from there to Ecuador. It is possible that the presence of *T. vivax* in Convento Village could be related to entry of cattle from some endemic areas. Other outbreaks of *T. vivax* in cattle have already been reported in countries of the region due to the high mobility of animals. Indeed, in the state of Zulia in Venezuela, an outbreak was declared 12 days after the introduction of Zebu cattle from Colombia without the corresponding sanitary controls or quarantine (Gonzatti et al., 2014; Simoes et al., 2009).

The presence of both pale mucosa and increase of temperature was observed in two of three TviCatL-PCR positive animals. Seven animals which showed pale mucosa were negative for TviCatL-PCR, possibly due to nutritional factors or previous unrecorded treatment. Reports indicate that anaemia could be an important appealing sign of trypanosomosis; for example, in cattle from dairy herds in Brazil, it was present in 46.66% of infected animals and 7.78% in healthy animals (Cuglovici et al., 2010). This clinical sign has also been recorded in sheep, which in experimental infections showed severe anaemia (Parra-Gimenez & Reyna-Bello, 2019). In the description of this process, the trans-sialidase enzymes trigger erytrophagocytosis by desialylating the glycophorin, causing anaemia during illness (Guegan et al., 2013). It has also been described as mechanical and biochemical damage of the erythrocyte membrane directly through the effect of the hemoflagellate during the parasitemia (Boada-Sucre et al., 2016).

The presence of *T. vivax* in the area of the outbreak could be explained by the occurrence of *Tabanu* (Jaimes-Dueñez et al., 2017), as it has been showed in a Colombian study (Jaimes-Dueñez et al., 2018). In addition, when the farms were visited, some management practices in cattle were seen, such as the reuse of syringes which may have contributed to iatrogenic transmission (Desquesnes, 2004).

Other haemotropic agents in the coastal region of Ecuador, that is *Anaplasma marginale* and *Babesia* spp. (Medina-Naranjo et al., 2017; Tana-Hernández et al., 2017) added to nutritional problems and stress, and could have caused the loss of enzootic balance in cattle from Convento Village. Similar cases have been reported in water buffaloes from Venezuela. Indeed, a trypanosomosis outbreak occurred in water buffaloes in 2015 during a stressful scarcity of green forage due to a drought season, along with concurrent infections with *Anaplasma* sp. and *Babesia* sp (Garcia et al., 2016).

5 | CONCLUSION

This study showed, for the first time, the presence of *T. vivax* in two herds in Ecuador and draw the attention about tripanosomosis in livestock in the coastal and jungle regions of Ecuador which have the optimal conditions for the parasite development (Medina-Naranjo et al., 2017). Therefore, the next step is to estimate the prevalence of *T. vivax* and risk factors associated, and characterize potential vectors for its transmission to determine whether epidemiological surveillance and control programs against trypanosomosis are necessary in Ecuador.

ACKNOWLEDGMENTS

The authors would like to thank all farmers who participated in the study. This study had the technical, scientific and financial support of the Universidad de las Fuerzas Armadas—ESPE, and was supported by the Academy of Research and Higher Education (ARES) through the Research for Development Project (PRD) entitled 'Establishment of a support platform for training and awareness raising, diagnosis and development of a strategy for the control of brucellosis and trypanosomiasis in Ecuador'.

CONFLICT OF INTEREST

None of the authors has financial or personal conflict of interest related to this study.

ETHICAL APPROVAL

The authors have a permit for intervention on the farms during the outbreak delivered by AGROCALIDAD, and authorization to use the information through the informed consent of farmers.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

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How to cite this article: Chávez-Larrea MA, Medina-Pozo ML, Cholota-Iza CE, et al. First report and molecular identification of *Trypanosoma (Duttonella) vivax* outbreak in cattle population from Ecuador. *Transbound Emerg Dis*. 2021;68:2422–2428. https://doi.org/10.1111/tbed.13906